

Original article

Linker for activation of T cells contributes to airway inflammation in an asthmatic mouse model

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Keywords: *asthma; linker for activation of T cells; T-lymphocytes*

Background Allergic asthma is associated with airway inflammation and hyperresponsiveness caused by dysregulated production of cytokines secreted by allergen-specific helper T-type 2 (Th2) cells. The linker for activation of T cells (LAT) is a membrane-associated adaptor protein, which has been shown to take part in regulating T cell receptor (TCR) signaling and T cell homeostasis. In this study, we established an asthmatic mouse model to examine the changes in LAT levels during allergic airway disease and the effects of LAT transgenic expression on airway inflammation.

Methods T cells from mouse lung tissues were isolated from allergen challenged (ovalbumin (OVA)) and control mice, and the purity of these isolated T cells was examined by fluorescence-activated cell sorter (FACS). Semi-quantitative RT-PCR and Western blotting were used to detect the expression of the LAT gene and LAT protein, respectively. After an intranasally administered mixture of pCMV-HA-LAT plasmid and Lipofectamine 2000, 24 hours before and 72 hours after allergen challenge, the BALF cell count and the differential cytologies were studied. In addition, IL-4 and IFN- γ levels in the BALF were determined by ELISA, and pathological changes in lung tissues were observed.

Results LAT protein and mRNA expression were decreased in lung T cells in a mouse model of allergen-induced airway disease. After intranasal administration of pCMV-HA-LAT, histopathological examination of the lungs showed that intervention with LAT overexpression prevented mice from developing airway inflammation, and the number of total cells, eosinophils, neutrophils, and lymphocytes in the BALF was reduced significantly compared with the OVA sensitized and challenged group. In addition, the Th2 cytokine IL-4 decreased, while the Th1 cytokine IFN- γ increased compared to the OVA sensitized and challenged group or the OVA sensitized group plus pCMV-HA treatment.

Conclusion This study demonstrates that LAT might effectively diminish Th2 cytokine responses, lung histopathological changes and lung inflammation to allergen challenge in a model of experimentally induced asthma.

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Asthma is a chronic inflammatory disorder of the airway in which T lymphocytes, eosinophils, mast cells and other inflammatory cells play a defensive role.^{1,2} Multiple investigations have demonstrated that an imbalance in Th1/Th2 cell types plays an important role not only in the pathogenesis of asthma, but also in the entire inflammatory process and even airway remodeling.^{3,4} In addition, there has been much interest focused on the role of cytokines secreted by allergen specific Th1/Th2 cells, such as IFN- γ and IL-12.⁵⁻⁸ These cytokines act as regulators of allergic inflammatory processes that can down-regulate Th2-biased immune responses in the respiratory tract, thereby protecting against the symptoms associated with allergies and asthma. Accordingly, the clinical potential for cytokine-based therapies to treat asthma is under investigation and these approaches include utilizing cytokines, anti-cytokine antibodies, or cytokine genes (especially the use of transient cytokine gene expression therapy). However, there is little known about the role of signaling proteins that participate in T cell development and differentiation during the pathogenesis of asthma.

The linker for activation of T cells (LAT) is a membrane-associated adaptor protein that is phosphorylated on multiple tyrosine residues upon T cell

receptor (TCR) cross-linking. Moreover, signaling through LAT to several common downstream signaling pathways has specific consequences for T cell development and differentiation.^{9,10} Several studies have shown that mutation of a single tyrosine residue in LAT can impede T cell development, which subsequently leads to the chronic overproduction of Th2 cytokines in large amounts.^{11,12} However, it remains unclear whether LAT plays a role in the pathogenesis of asthma.

The aim of this study was to investigate whether the process of asthma pathogenesis is dependent on LAT activation as measured by the effects of LAT on T cell differentiation. Hence, an asthmatic mouse model was established to investigate the expression of LAT in lung T

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cells by semi-quantitative RT-PCR and Western blotting analysis as compared to control animals. After transfection with the LAT gene intranasally, the expression of LAT in lung tissue, the level of IL-4, and the amounts of IFN- γ in the bronchoalveolar lavage fluid (BALF) were determined. In addition, changes in lung histology were observed.

METHODS

Animals

Female, 6 to 8-week-old wild-type BALB/C mice from the Shanghai Xipuer-Bikai Experimental Animal Company (China) were maintained under pathogen-free conditions at the animal center of Xinhua Hospital, Shanghai and were age- and sex-matched within each experiment. All procedures were approved by the Xinhua Hospital Animal Care and Use Committee. Eighty-eight mice were used for identification of T lymphocyte purity from mouse lung tissues and the expression of LAT in the T cells of the lungs. Another 28 mice were used for the transfection with the LAT gene.

Establishment of an experimental allergic asthma mouse model

The allergen challenged group was immunized by intraperitoneal (i.p.) administration of 50 μ g ovalbumin (OVA, Beijing Dingguo Company, Beijing, China) absorbed to 100 μ g aluminum hydroxide (Shanghai Experiment Reagent Co., China) on day 0, 7, and 14, followed by an aerosol challenge with nebulized OVA (2%, 800 mg OVA in 40 ml PBS) for 30 minutes once a day on day 15–20. In the negative control group, OVA was replaced by 0.9% sodium chloride. On day 21, mice were exsanguinated and sacrificed, the BALF was collected, and the trachea and lungs were removed.

T lymphocytes isolation and purity

Pneumocytes were digested in a collagenase I and DNase (Sigma, St. Louis, MO, USA) solution that was passed through a sterile wire mesh. Mononuclear cells were isolated by a continuous 30% Percol gradient (Pharmacia Chemical, St. Louis, MO, USA), then incubated with a panel of mouse mAbs, including mAbs against CD8 (B9.8), CD19 (FMC63), CD11b (NIH11b-1), CD14 (63D3), CD16 (3G8), MHC class II (IVA12), erythrocytes (glycophorin, 10F7), and platelets (37F9-E7). mAb against CD45R0 (UCHL-1) was added for the isolation naive CD4⁺ T cells. Ab-bound cells were subsequently removed by incubation with anti-mouse IgG-conjugated magnetic beads according to the manufacturer's recommendations (Dynal Biotech Beijing Ltd, Beijing, China). Unbound cells (naive CD4⁺ T cells) were collected, and T cell purity was at least 95% as determined by flow cytometry.

Reverse transcription-polymerase chain reaction

Total RNA from lung CD4⁺ T cells was extracted from 1–5 $\times$ 10⁶ cells using Trizol reagent (Invitrogen Carlsbad, CA, USA). One sample included CD4⁺ T cells isolated

from two mice lungs, and there were 15 samples in the asthmatic and control groups respectively. Total RNA was reversely transcribed to generate first-strand complementary DNA (cDNA), and the resulting cDNA was amplified by polymerase chain reaction (PCR). The primer sequences for LAT were 5' TGTGCCTAGCAA CCCTGACC 3' (sense) and 5' TGGACCCCAAAGTG ACAATAAG 3' (antisense). For β -actin, the primer sequences were 5' AAGTGTGACGTTGACATCCGTAA AG (sense) and 5' CAGCTCAGTAACAGTCCGCTA GA 3' (antisense). PCR amplification was performed as follows: 94°C for 3 minutes, and then 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, followed by 72°C for 10 minutes. The integral optical density (IOD, equal to the area $\times$ optical density) of the electrophoresis strips was counted after agarose gel electrophoresis. The ratio of LAT/ β -actin was determined as the relative content of LAT mRNA.

Western blotting

CD4⁺ T cells (one sample included T cells isolated from two mice lungs, and there were 7 samples in the OVA challenged and control groups) were washed with PBS and solubilized with 1 $\times$ loading buffer, and boiled for 5 minutes in 100°C water. For blotting, 20 μ l per sample was resolved by 15% SDS-PAGE, and then transferred to PVDF membranes, blocked with 5% dry milk in PBS-T (PBS/0.1% Tween 20) before incubation overnight with a rabbit anti-mouse LAT antibody (1:1000, Bioscience, San Jose, CA, USA). After washing with PBS-T, blots were incubated in goat anti-rabbit IgG conjugated to horseradish-peroxidase (HRP, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed, and developed with enhanced chemiluminescence (Pierce, USA). The expression of the β -actin protein was detected as a control. The antibodies used were rabbit anti-mouse β -actin monoclonal antibody (1:1000, Bioscience) and goat anti-rabbit IgG conjugated to HRP (1:1000, Santa Cruz Biotechnology). The trachea and pneumocytes were homogenized, centrifuged, the supernatant was collected, and then the procedure was continued the same as above.

Cloning and recombination

The cDNA for the mouse LAT gene was amplified by polymerase chain reaction (PCR). The primers used were 5' ATGGAAGCAGACGCCTTGAGCC 3' (sense) and 5' CACCCAGCAAGTCCAGTTTCAGG 3' (antisense). The resulting fragment was inserted into the pCMV-HA plasmid (Clontech Laboratories, Inc, Palo Alto, CA, USA) for eukaryotic expression. The recombinant plasmid was verified by DNA sequencing and confirmed by alignment with the corresponding Genebank entry (NM010689).

Transfection with the LAT gene

Mice were divided into the 4 following groups (A–D) with 7 mice per group: A, negative control group; B, OVA challenged group with no interventional method; C, OVA challenged mice treated intranasally on days 14 and 17 with a 50 μ l mixture of pCMV-HA plasmid and Lipofectamine 2000 (Invitrogen); D, OVA challenged

mice treated intranasally on days 14 and 17 with a 50 μ l mixture of pCMV-HA-LAT plasmid and Lipofectamine 2000. On day 21, all mice were exsanguinated and sacrificed, BALF was collected, and the lungs were removed.

Bronchoalveolar lavage and lung histology

Mice were sacrificed within 24 hours of the last challenge, the chest cavity was opened, and the lungs were lavaged with PBS via the trachea. The BAL sample was collected by cytocentrifugation, while the remaining cells were fixed in ethanol (50%). The total number of cells collected was determined by counting the cells in a Burkert chamber. After cytocentrifugation, the cells from the bronchoalveolar lavage were stained with hematoxylin and eosin (H&E) and the percent distribution for the different cell-types was determined. The lungs were fixed in 10% formalin, embedded in paraffin wax, routinely processed, sectioned to 5 μ m thickness, and stained with H&E.

Measurement of cytokines

Levels of IL-4 and IFN- γ were quantified in the supernatants of BAL fluids by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (RapidBio Lab, Calabasas, CA, USA).

Statistical analysis

The statistical analysis was performed using SPSS 11.0 program (SPSS, USA). The paired-sample *t* test and one-way analysis of variance (ANOVA) were used to test for significant differences in measured variables between groups. A *P* value less than 0.05 was considered statistically significant.

RESULTS

LAT mRNA and protein expression in lung T cells of asthmatic and control mice

RT-PCR (Figure 1A) and Western blotting analysis (Figure 1B) revealed that LAT expression was decreased in the lung T cells of asthmatic mice compared to control mice. These findings suggested that alterations in LAT levels were associated with the asthmatic phenotype.

LAT expression after administration of pCMV-HA-LAT

The pCMV-HA vector, encoding the complete cDNA of mouse LAT was constructed and termed pCMV-HA-LAT. The expression of the LAT transgene was verified by transfecting mouse lymphocytes with pCMV-HA-LAT followed by Western blotting (data not shown). To determine the effect of pCMV-HA-LAT administration on an experimental murine model of allergic asthma, mice were divided into the four groups as shown in Figure 2A. The expression of the LAT protein in the mouse trachea and pneumocyte was dramatically increased after intranasally administered pCMV-HA-LAT (Figure 2B).

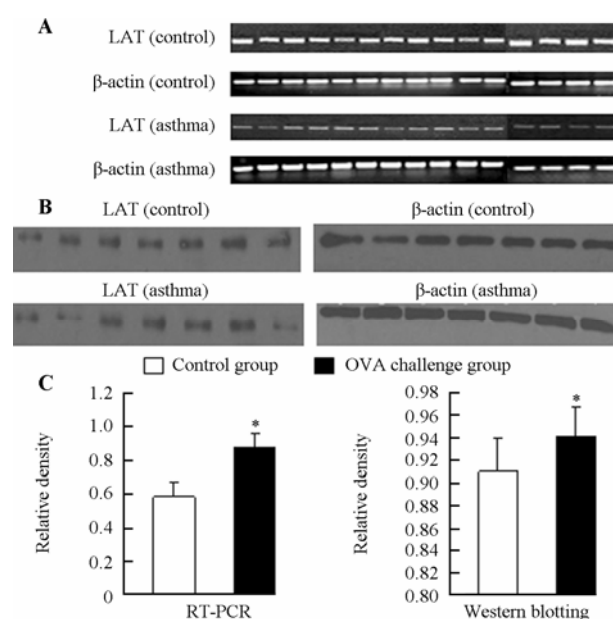


Figure 1. Levels of LAT expression in lung T cells from OVA challenged and control mice. **A:** RT-PCR analysis of LAT mRNA expression. **B:** Western blotting analysis of LAT protein. **C:** Densitometric analyses are presented as the relative ratio of LAT to β -actin. Data represent mean $\pm$ standard error from 15 samples per group for RT-PCR analysis and 7 samples per group for Western blotting analysis. **P* < 0.05 versus saline inhalation.

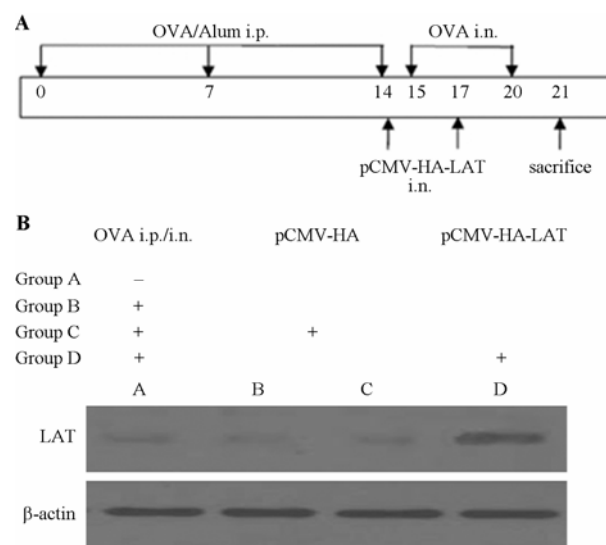


Figure 2. LAT expression after administration of pCMV-HA-LAT. **A:** Experimental outline. **B:** Expression of LAT protein in mouse trachea and pulmons as examined by Western blotting among the four groups. LAT protein can be detected in each group. After intranasal (i.n.) administration of pCMV-HA-LAT (D group), the expression of LAT protein in the lung was increased.

Effect of LAT expression on lung histology

Lung inflammation was examined in formalin-fixed, H&E stained lung sections from all four groups. The pCMV-HA-LAT treated group exhibited reduced epithelial damage and less infiltration of mononuclear cells in the interstitial and peribronchovascular regions (Figure 3D) compared with the control group receiving pCMV-HA.

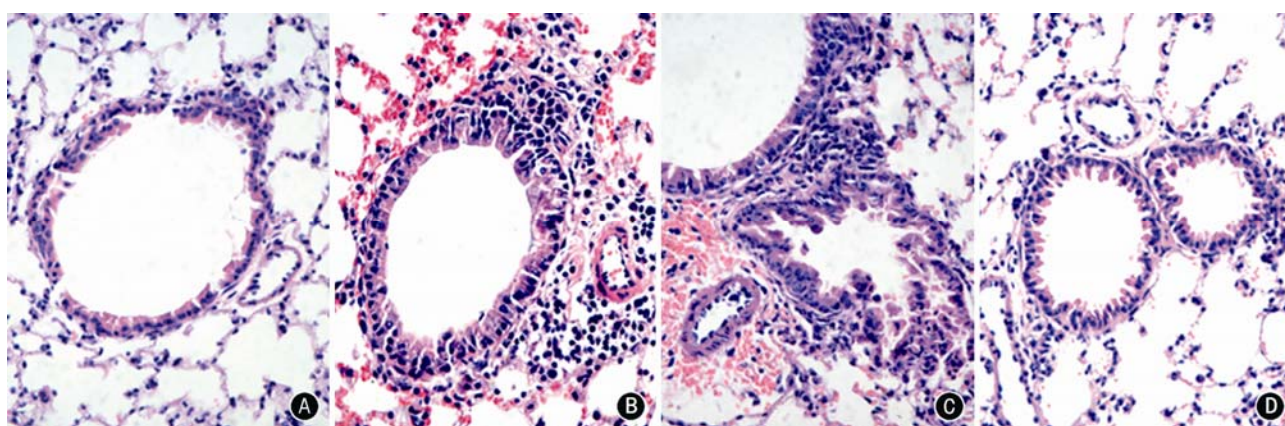


Figure 3. Lung histopathological changes were decreased by pCMV-HA-LAT expression. Mouse lungs from each experimental group were removed, fixed in formalin, and then were embedded in paraffin, sectioned, and stained with H&E as described in the Methods section. **A:** non-sensitized mice; **B:** OVA-challenged mice; **C:** OVA challenged mice treated with pCMV-HA; **D:** OVA challenged mice treated with pCMV-HA-LAT. OVA challenged mice developed a strong peribronchovascular infiltrate containing numerous eosinophilic granulocytes after OVA challenge. Treatment with pCMV-HA-LAT (**D**) reduced epithelial damage and infiltration of mononuclear cells in the interstitial and perobronchovascular regions compared with the group receiving pCMV-HA (**C**).

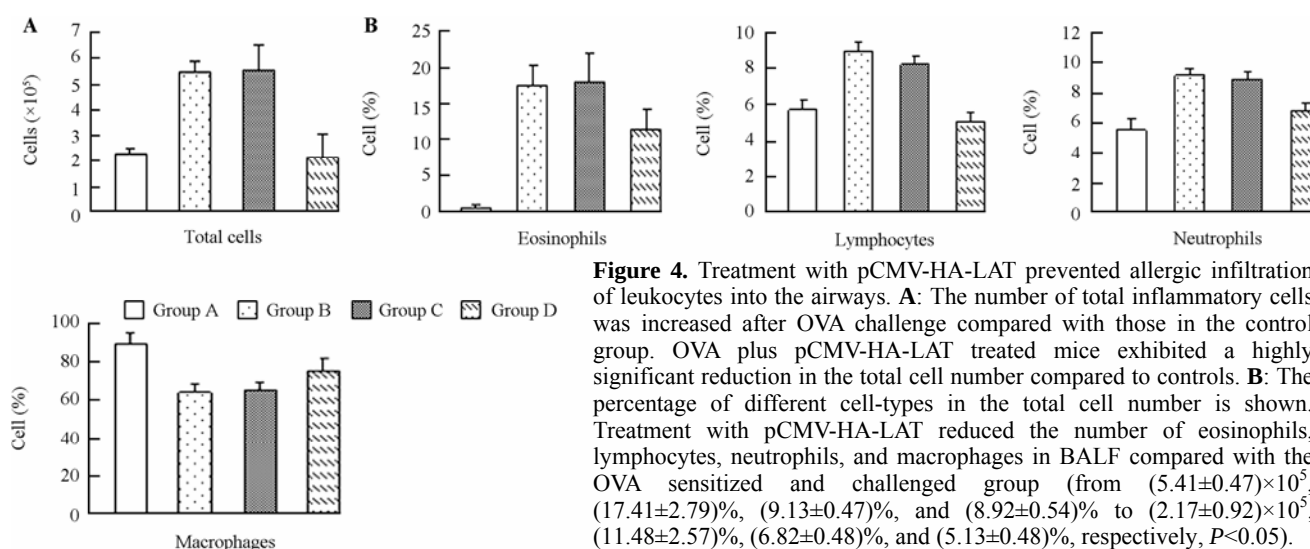


Figure 4. Treatment with pCMV-HA-LAT prevented allergic infiltration of leukocytes into the airways. **A:** The number of total inflammatory cells was increased after OVA challenge compared with those in the control group. OVA plus pCMV-HA-LAT treated mice exhibited a highly significant reduction in the total cell number compared to controls. **B:** The percentage of different cell-types in the total cell number is shown. Treatment with pCMV-HA-LAT reduced the number of eosinophils, lymphocytes, neutrophils, and macrophages in BALF compared with the OVA sensitized and challenged group (from $(5.41 \pm 0.47) \times 10^5$, $(17.41 \pm 2.79)\%$, $(9.13 \pm 0.47)\%$, and $(8.92 \pm 0.54)\%$ to $(2.17 \pm 0.92) \times 10^5$, $(11.48 \pm 2.57)\%$, $(6.82 \pm 0.48)\%$, and $(5.13 \pm 0.48)\%$, respectively, $P < 0.05$).

(Figure 3C) or the OVA-sensitized and challenged group (Figure 3B). Histopathological examination of the lungs from OVA-sensitized mice revealed a pleiomorphic peribronchovascular infiltrate consisting of eosinophils, lymphocytes, and neutrophils that were not seen in control mice. However, intervention with the LAT transgene prevented mice from developing this pulmonary infiltration.

Treatment with pCMV-HA-LAT prevented allergic infiltration of leukocytes into the airways

BALF was collected on day 21. Meanwhile, the numbers of total cells (Figure 4A), eosinophils, and neutrophils (Figure 4B) were increased after OVA sensitization and challenge compared with those in the control group. Treatment with pCMV-HA-LAT reduced the number of total cells (Figure 4A), eosinophils, lymphocytes, and neutrophils (Figure 4B) in the BALF compared with the OVA-sensitized and challenged groups (from $(5.41 \pm 0.47) \times 10^5$, $(17.41 \pm 2.79)\%$, $(9.13 \pm 0.47)\%$, and $(8.92 \pm 0.54)\%$ to $(2.17 \pm 0.92) \times 10^5$, $(11.48 \pm 2.57)\%$,

$(6.82 \pm 0.48)\%$, and $(5.13 \pm 0.48)\%$, respectively, $P < 0.05$), whereas alveolar macrophages were increased ($(64.42 \pm 4.89)\%$ to $(75.31 \pm 6.62)\%$, $P < 0.05$, Figure 4B). These results demonstrated that pCMV-HA-LAT treatment significantly attenuated lung eosinophilia.

pCMV-HA-LAT promoted a Th1 cytokine profile in the lungs

During allergic inflammation, recruited inflammatory cells, airway epithelial cells, and resident airway macrophages were activated and released cytokines promoting a Th2 type immune response in the lungs. In turn, these cytokines maintained this inflammatory process by autocrine and paracrine mechanisms. To determine whether pCMV-HA-LAT treatment could influence cytokines release, we examined the concentrations of IL-4 and IFN- γ in BAL fluid. Because T cells were reduced after pCMV-HA-LAT treatment, the Th2 cytokine IL-4 was decreased compared to the OVA challenged group and the OVA plus pCMV-HA-treated group ((61.86 ± 6.68) , (85.73 ± 7.83) , and (88.07 ± 4.21)

pg/ml, respectively, $P < 0.05$, Figure 5A). In addition, the Th1 cytokine IFN- γ was increased compared to the OVA challenged group or the OVA plus pCMV-HA-treated group ((106.20 $\pm$ 13.63), (84.21 $\pm$ 17.62), and (86.96 $\pm$ 10.28) pg/ml, respectively, $P < 0.05$, Figure 5B). Thus, pCMV-HA-LAT treatment *in vivo* can influence the cytokine milieu in targeted tissues.

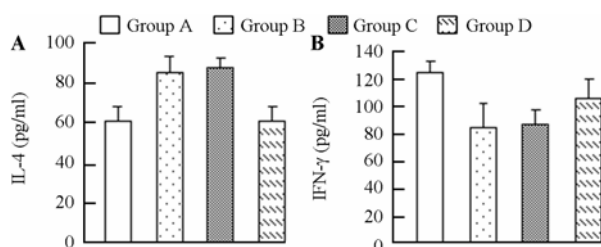


Figure 5. Treatment with pCMV-HA-LAT altered cytokines production. Cytokine concentrations were measured in a cell-free BAL fluid. **A:** IL-4 was decreased in the OVA plus pCMV-HA-LAT-treated group compared to the OVA-sensitized and challenged group or the OVA plus pCMV-HA-treated group. **B:** IFN- γ was increased compared to the OVA-sensitized and challenged group or the OVA plus pCMV-HA treated group.

DISCUSSION

In this study, the protein and mRNA expression of LAT were decreased in lung T cells from a mouse model of OVA-induced asthma. These findings suggest that alterations in LAT levels are associated with an asthmatic phenotype.

LAT is a transmembrane adapter protein that couples the T cell receptor to downstream signaling effectors. This adapter protein is a dual substrate for Lck and Syk non-receptor tyrosine kinases in T lymphocytes,¹³ and has been identified as an integral transmembrane protein of 36–38 kDa. LAT possesses a single region of about 20 hydrophobic amino acids that constitutes the transmembrane segment and is expressed in the membrane of T lymphocytes, NK and mast cells, platelets, and pre-B cells.¹⁴ Similar to the majority of adapter proteins, LAT lacks any enzymatic or transcriptional activity. LAT contains in its amino acid sequence 9 conserved tyrosine residues that, upon phosphorylation, are potential docking sites for different signaling proteins. Indeed, from the initial studies examining this adaptor protein, it was demonstrated that four distal tyrosine residues (Y132, Y171, Y191, and Y226) are essential for the association with critical signaling molecules. Thus for TCR signals transduction, tyrosines 7, 8, and 9 (Y171, Y191, and Y226) are responsible for binding to the SH2 domain of Grb2, while tyrosine 6 (Y132) binds upon its phosphorylation to the C-terminal SH2 domain of PLC- γ 1, while the Gads-SLP-76 complex binds to phosphorylated tyrosines 7 and 8 (Y171 and Y191).^{15,16} Upon their binding to LAT, these proteins can themselves be subsequently activated by tyrosine phosphorylation.

Early studies using transformed T cell lines suggested that LAT acts primarily as a positive regulator of TCR signaling. Moreover, the partial or complete inhibition of T cell development observed in several mouse lines harboring mutant forms of LAT has been consistent with that view. More recently, LAT “knock-ins” harboring point mutations in the four COOH-terminal tyrosine residues were found to develop lymphoproliferative disorders involving polyclonal T cells that produced high amounts of T helper type-2 (Th2) cytokines. This phenotype promoted Th2 differentiation and caused tissue eosinophilia, as well as rapid massive maturation of plasma cells secreting immunoglobulins of the E and G1 isotypes.^{11,12}

The T cell, especially the CD4⁺ T cell, is one of the key cell-types involved in the pathogenic mechanisms of asthma. The imbalance of the cytokines secreted by Th1/Th2 cells is thought to be a core pathogenesis of pulmonary hypersensitivity and asthma. In addition, this imbalance plays a pivotal role not only during an asthmatic attack, but also in the entire inflammatory process, even during airway remodeling.^{3,4} But the detailed mechanisms underlying allergic asthma are extremely complicated and many processes have not been elucidated, further investigations are still needed. The unexpected findings that LAT regulates TCR signaling and T cell homeostasis imply that LAT may take part in the pathogenesis of asthma.

A significant finding of this study was that a single administration of LAT delivered as a cDNA in an expression vector induced a Th1-biased response in the lungs and significantly increased production of IFN- γ , while also reducing production of IL-4. In the lungs, LAT administration reduced airway eosinophilia and goblet cell hyperplasia, but it induced a Th1-biased mononuclear cell inflammatory infiltrate in the airways. The generation of the Th1-biased inflammatory infiltrate was primarily attributed to the presence of LAT, because administration of a control plasmid had no effect on airway inflammation. These results indicate that administration of LAT into the lungs of asthmatic mice can effectively down-regulate previously established Th2-dominated inflammatory responses in the respiratory tract.

Recently, the identification of methods to prevent, control, or even reverse the process of Th2 immunodeviation has become a focus for the development of new strategies to control asthma. Specific attempts to alter the Th1/Th2 balance by enhancing Th1 and abrogating Th2 responses have been the major aim of immunological approaches for the prevention and treatment of asthma. Furthermore, induction of Th1 responses may have a direct suppressive effect on Th2-mediated inflammatory processes. Currently, the most direct approaches include administration of cytokines that will induce activation of Th1 pathways (e.g. IFN- γ ,^{5,6} IL-12,^{7,8} and IL-18^{17,18}) or by blocking antibodies that inhibit the effect of

Th2-related cytokines (e.g. anti-IL-4,¹⁹ anti-IL-5²⁰). In this paper, we showed that overexpression of the LAT protein, which is an adapter protein serving an important role in T cell development, activation, and homeostasis, could also reverse the Th1/Th2 imbalance existing in this asthmatic mouse model.

However, the mechanism responsible for LAT down-regulation in the T cells of the asthmatic mouse is unclear. Nevertheless, the present results examining the administration of LAT suggest that LAT may take part in the pathogenesis of asthma. As a transmembrane scaffolding protein in a complex signal transduction pathway, LAT is affected by both upstream signaling proteins, including members of the Src and Syk family of intracellular tyrosine kinases, and downstream signaling proteins, including SLP-76, PLC- γ , Grb2, and others. Thus, the levels of these proteins in the asthmatic mouse may influence the expression and function of LAT.

In summary, we have shown that LAT expression was decreased in the lung T cells from a mouse model of asthma. Moreover, LAT administration resulted in a diminished allergic asthma phenotype in the OVA challenged mouse model, which was characterized by persistently elevated production of Th2-like cytokines and pulmonary inflammation. However, the mechanisms responsible for LAT-mediated attenuation of allergic asthma remain to be elucidated.

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